In Vitro Effect of Erythropoietin on the Spleen of the Polycythemic Mouse. III: Limited Exposure to Erythropoietin and Actinomycin D

By Yasusada Miura, Hideaki Mizoguchi, Fumimaro Takaku and Kiku Nakao

Since Jacobson et al., 1 Alpen et al. 2 and Ersvē 3 have suggested that the erythropoietin acts on the erythropoietic stem cells and causes their differentiation, this hormone has been utilized as a tool to study the control mechanism of steady-state erythropoiesis. Krantz et al., 4,5 Dukes et al. 6 and Gallien-Lartigue et al. 7 have reported several studies on the response in vitro of starved rat bone marrow cells to erythropoietin in relation to the RNA synthesis.

In the present study, the effects of an initial exposure to erythropoietin on the further development of the erythroid cells in the spleen explants derived from polycythemic mice 8 were observed. In some cultures, both actinomycin D and erythropoietin were added in order to study the effect of the antibiotic on the erythropoietin-induced erythroid cell differentiation.

MATERIALS AND METHODS

Tissue culture of Polycythemic Mouse Spleen

Animals. Young adult female dd-mice weighing about 20 g. were made plethoric by intravenous infusion of 1.5 ml. of a 50 percent red cell suspension taken from the homologous mice. Only mice with a hematocrit over 65 percent were sacrificed from 4–6 days after transfusion. Erythroblasts were rarely found in the spleens under these conditions.

Tissue culture methods are the same as previously reported. 8 In a series of experiments, inactivated calf serum of the same lot from the Chiba Serum Institute was used.

The NCTC 109 solution was prepared from the dried material supplied from the Difco Co. Erythropoietin was obtained from the urine of anemic patients according to the method of alcohol-acetone precipitation. The lyophilized erythropoietin contained an activity 14 U/mg. calibrated in starved rat assay against Erythropoietin Standard A. 9 Erythropoietin was dissolved in the normal saline to obtain an activity of 20 U/ml. and filtered through a Millipore filter prior to use. Actinomycin D was supplied by Merck, Sharp and Dohme Co. and diluted in the normal saline to obtain a concentration of 0.5 μg/ml. prior

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Table 1.—Effect of Medium Replacement on the Heme Synthesis of the Polycythemic Mouse Spleen Explants Incubated with 0.5 U/ml. Erythropoietin for Throughout 48 Hours

<table>
<thead>
<tr>
<th>Time of Medium Replacement (h)</th>
<th>No. of Experiments</th>
<th>59Fe Uptake Into Heme X 10^{-3} /mg. Spleen (M±Se)</th>
</tr>
</thead>
<tbody>
<tr>
<td>without replacement</td>
<td>5</td>
<td>325 ± 81.3</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>263 ± 28.5</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>329 ± 97.7</td>
</tr>
<tr>
<td>12</td>
<td>8</td>
<td>403 ± 68.5</td>
</tr>
<tr>
<td>24</td>
<td>9</td>
<td>303 ± 55.8</td>
</tr>
</tbody>
</table>

To use. Three microcuries of 59FeCl3 was added in each tube six hours prior to the termination of the incubation. The radioactive heme was extracted by acid methylethyl-ketone, placed on a steel planchet, and was thoroughly dried. The radioactivity of β-ray from 59Fe was counted in a gas flow counter of about 20 cpm background. Preliminary experiments showed that the heme concentration obtained in the present experiments did not cause any self-absorption of the β-ray from 59Fe.

In the experiments in which the spleen explants were exposed to erythropoietin for certain periods, the entire incubation medium was exchanged at the indicated time of incubation. All the medium of the control culture tubes was also changed at the same time. Spleen fragments which were exposed to erythropoietin throughout 48 hour incubation period served as the control cultures.

Morphologic observation was carried out from a stamp specimen of the explants. Numbers of erythroblasts among 1000 nucleated cells were counted. According to the numbers of erythroblasts, the experimental specimens were arbitrarily classified into four grades as indicated in Tables 2 and 3.

Experiments on Mouse Reticulocytes

Young adult female dd-mice weighing about 20 g. were used for the experiments. Approximately 0.5 ml. of venous blood was taken from the orbital venous plexus. Four days after the bleeding, peripheral reticulocytes increased close to 20 percent of red blood cells. Mice were sacrificed by cervical dislocation and the cardiac blood was suspended in heparinized saline, washed once with the normal saline and resuspended in the incubation medium which contains 20 percent calf serum and 80 percent NCTC 109 solution. Each experiment was carried out in triplicate.

Five-tenths ml. of reticulocyte suspension was placed in a tissue culture tube and 0.5 ml. of the incubation mediums containing erythropoietin, actinomycin D or both was added. The tube without erythropoietin or actinomycin D served as the control. Three microcuries of 59FeCl3 dissolved in 0.1 ml. of NCTC 109 solution was added from the beginning of the incubation. Then, the culture tubes were incubated at 37 C. for four hours. Upon completion of the incubation, the cells were washed with normal saline three times.

The extraction of heme from the washed cells was carried out by the same method as described above. The radioactivity in the extracted heme solution was counted in a well-type scintillation counter of about 90 cpm background.

RESULTS

Effects of Medium Replacement

No significant difference was observed in the heme synthesis rate of polycythemic mouse spleens among the control experiments in which the incubation medium was changed at 3, 6, 12 or 24 hours of incubation or unchanged throughout the 48-hour incubation (Table 1).
ERYTHROPOIETIN AND POLYCYTHEMIC MOUSE

Table 2.—Effect of Limited Exposure to Erythropoietin on Radioiron Incorporation in the Heme of Polycythemice Mouse Spleen Explants In Vitro

<table>
<thead>
<tr>
<th>Hours of Exposure to Erythropoietin (0.5U/ml.) (Hrs.)</th>
<th>No. of Experiments</th>
<th>% Rate of Heme Synthesis Compared to the Control Experiments**†† (M ± Se)</th>
<th>No. of Cases Showing Erythroblasts†§</th>
<th>Total</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13</td>
<td>2.6 ± 0.5</td>
<td>9</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>20.4 ± 1.9</td>
<td>0</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>53.4 ± 18.7</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>4</td>
<td>69.0 ± 8.1</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>24</td>
<td>5</td>
<td>74.6 ± 10.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>48</td>
<td>17</td>
<td>100.0 ± 0.0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

*One specimen consists of spleen explants collected from two different culture tubes.
††Fe uptake into the heme per mg. net weight of the same spleen incubated with erythropoietin for 48 hours = 100%.
§The erythroblasts were counted in the explants after 48 hours incubation.
||No. of Erythroblasts per 1000 nucleated cells at 48th hour of incubation are designated as follows: -- 0, + 1–30, ++ 31–100, +++ 100–

Effects of Limited Exposure to Erythropoietin on The Erythroid Cell Differentiation in Polycythemic Mouse Spleen (Table 2)

A considerable increase in heme synthesis was observed in the spleen fragments exposed to 0.5 U/ml. erythropoietin for three hours at the beginning of the culture period. The heme synthesis rate increased in proportion to the increase of the contact time to erythropoietin up to 12 hour exposure.

Heme synthesis rate in the explants exposed to erythropoietin for 12 or 24 hours reached 69.5 percent and 75 percent respectively of the control explants which were exposed to erythropoietin throughout the 48-hour incubation. Large erythroblasts appeared in the explants at the 24th and small erythroblasts at the 48th hour in the explants exposed to erythropoietin for each 3, 6, 12, 24 and 48 hours. No erythroblasts, however, appeared in the control explants incubated for 48 hours without erythropoietin except in one case in which only one erythroblast per 1000 nucleated cells was observed (Table 2).

Effect of Actinomycin D on The Spleen Explants

Addition of 0.01 µg/ml of actinomycin D from the beginning of incubation completely suppresses heme synthesis as well as an appearance of erythroblasts in the explants incubated with 0.5 U/ml. erythropoietin.

The effect of actinomycin D was proportional to the time of exposure of the cells to the drug during the first 24 hours of culture. In other words, delayed exposure of cells to actinomycin resulted in increased levels of heme synthesis (Table 3).

Also in this experiment, morphologic observation showed a parallelism between the appearance of erythroblasts and heme synthesis (Table 3). Although the incubated cells showed a slight structural derangement at the 48th hour of incubation, erythroblasts were easily recognizable from the stamp specimen.
Table 3.—Effect of Actinomycin D on the Heme Synthesis in the Spleen Explants from Polycythemic Mouse Spleen In Vitro

<table>
<thead>
<tr>
<th>Time of Addition of Actinomycin D (0.01 μg./ml.) (Hrs. of Incubation)</th>
<th>No. of Experiments</th>
<th>% Rate of Heme Synthesis Compared with The Control Experiments* (M±5e)</th>
<th>No. of Cases Showing Erythroblasts†</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>8</td>
<td>2.9 ± 0.5</td>
<td>5</td>
</tr>
<tr>
<td>12</td>
<td>4</td>
<td>13.1 ± 2.2</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>4</td>
<td>45.4 ± 4.1</td>
<td>0</td>
</tr>
<tr>
<td>control</td>
<td>8</td>
<td>100.0 ± 0.0</td>
<td>0</td>
</tr>
</tbody>
</table>

In each experiment, 0.5 U/ml. of erythropoietin is added in the medium throughout the experiment.

*35Fe uptake into heme per mg. wet weight of each spleen incubated without actinomycin D = 100%.

†The heme synthesis was measured from the explants after 48 hours incubation.

‡The erythroblasts were counted in the explants after 48 hours incubation. The numbers of Erythroblasts per 1000 nucleated cells at 48th hour of incubation are designated as follows: − 0, + 1 ~ 30, ++ 31 ~ 100, +++ 100 ~

Effect of Erythropoietin and Actinomycin D on The Heme Synthesis of Reticulocytes

No significant effect of 0.25 U/ml. or 0.5 U/ml. erythropoietin was observed on the radio-iron incorporation into heme in the mouse reticulocytes.

Actinomycin D in the concentration 0.01 μg./ml. or 0.1 μg./ml. did not affect the heme synthesis of reticulocytes incubated either with or without 0.5 U/ml. of erythropoietin.

DISCUSSION

In the present study, a significant response to erythropoietin was already observed when the incubated polycythemic mouse spleen was exposed to erythropoietin for 3 hours. Considering the necessary period to the adaptation of spleen explants to the tissue culture medium, it seems that actually a contact period shorter than three hours would be necessary to evoke stem-cell response to erythropoietin. To find a minimal necessary contact time to respond to erythropoietin in this system, an intensive investigation with preincubation of the tissue will be required.

A parallelism was observed between the number of erythroblasts and the heme synthesis rate in the spleen explants incubated with erythropoietin. Therefore, the increase in heme synthesis rate is considered to indicate the number of erythroblasts which are differentiated from stem cells during the incubation. The heme synthesis and the appearance of erythroblasts increased in proportion to the contact time of erythropoietin in the earlier period of incubation. It is to be noted that the heme synthesis reached approximately 70 percent of the control experiments when the tissue fragments were exposed to erythropoietin for 12 hours.

On the other hand, the experiments on the reticulocytes showed that the erythropoietin has no stimulatory effect on the heme synthesis by reticulocytes. From these results, it is strongly suggested that after a short-time contact of
the polycythemic mouse spleen to erythropoietin, a large population of erythropoietin-responsive stem cells starts to differentiate into erythroblasts and to complete such specific enzyme and protein synthesis as Nakao et al. have reported using the polycythemic mouse spleen.

The increase in the number of erythroblasts and heme synthesis is proportional to the contact time to erythropoietin until the 12th hour. This would be explained by the hypothesis that a limited group of stem cells is responsive to erythropoietin and the number of those cells would be kept in a steady-state from the "dormant" stem cells. Therefore, the present dose of erythropoietin could mobilize the majority of stem cells which is responsive to erythropoietin in 12 hours.

Actinomycin D is an antibiotic which is commonly used to inhibit RNA synthesis. It also may inhibit the synthesis of DNA and other aspects of cell metabolism, particularly at higher doses. The dose in the present experiment, 0.01 μg./ml., is much smaller than that used in the experiments in vitro.

Our preliminary experiments showed that 0.01 μg./ml. was the minimal dose that completely suppressed heme synthesis of spleen explants in this system. From the present experiments, it is to be noted that a considerable amount of heme synthesis and appearance of erythroblasts occur when actinomycin D is added at the 12th or 24th hour of incubation. A parallelism between the appearance of erythroblasts and the heme synthesis rate was also observed in this series of experiments. Therefore, it is probable that some of the population of erythropoietin-responsive stem cells acted upon by erythropoietin have become "resistant" to actinomycin D by the 12th hour of incubation and the response has reached half of the whole population at the 24th hour of incubation. The heme synthesis of reticulocytes is not affected by actinomycin D in the present concentration. Reissman and Ito also reported on the inability of 2 μg./ml. of the drug to alter the in vitro radio-iron uptake of reticulocytes. From the present observations, action of actinomycin D in our in vitro system is suggested to inhibit a new RNA synthesis related to the erythropoietin-induced differentiation as well as general metabolic process. The antibiotic, however, does not affect the heme synthesis after the intracellular synthetic apparatus has been completed as shown by results obtained with the reticulocytes. These results are compatible with the data on the in vivo erythropoietic response in polycythemic mice reported by Reissman and Ito. They applied small doses of actinomycin D after injection of erythropoietin.

Gallien-Lartigue and Goldwasser have reported the action of actinomycin D on the rat marrow suspension incubated for 32 hours with erythropoietin. According to their data, the dose of actinomycin D is much higher than that used in the present experiments. The depression of heme synthesis due to actinomycin D is evident. However, the time of addition of the drug at the 8th hour is considered to correspond to the 24th hour of our 48 hour incubation. Therefore, their results might be showing a cytotoxic effect on the drug as well as specific effect on mRNA synthesis.
SUMMARY

1. Heme synthesis and appearance of erythroblasts were observed when the spleen fragments from the polycythemic mouse spleen were exposed to erythropoietin only in the earlier period of incubation. The heme synthesis and the number of erythroblasts were increased in proportion to the exposure time to erythropoietin up to 12 hours. Twelve hours exposure to erythropoietin evoked about 70 percent of the maximally stimulated control heme synthesis.

2. The appearance of erythroblasts and the heme synthesis was completely blocked by the addition of 0.01 μg./ml. of actinomycin D. Some population of differentiated erythroblasts became resistant to actinomycin D which was added at 12th and 24th hour of incubation.

3. The heme synthesis within reticulocytes from bled anemic mice were not affected by these doses of erythropoietin, actinomycin D or both of them.

4. The results were discussed in connection with the mechanism of erythropoietin on stem cells.

REFERENCES


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